Gemcitabine synergizes with immune checkpoint inhibitors and overcomes resistance in a preclinical model and mesothelioma patients

Tallón de Lara, Paulino ; Cecconi, Virginia ; Hiltbrunner, Stefanie ; Yagita, Hideo ; Friess, Martina ; Bode, Beata ; Opitz, Isabelle ; Vrugt, Bart ; Weder, Walter ; Stolzmann, Paul ; Felley-Bosco, Emanuela ; Stahel, Rolf A ; Tischler, Verena ; Britschgi, Christian ; Soldini, Davide ; van den Broek, Maries ; Curioni Fontecedro, Alessandra

Abstract: PURPOSE Combination of immune checkpoint inhibitors with chemotherapy is under investigation for cancer treatment. EXPERIMENTAL DESIGN We studied the rationale of such a combination for treating mesothelioma, a disease with limited treatment options. RESULTS The combination of gemcitabine and immune checkpoint inhibitors outperformed immunotherapy alone with regard to tumor control and survival in a pre-clinical mesothelioma model; however, the addition of dexamethasone to gemcitabine and immune checkpoint inhibitors nullified the synergistic clinical response. Further, treatment with gemcitabine plus anti-PD-1 resulted in an objective clinical response in two mesothelioma patients, who were resistant to gemcitabine or anti-PD-1 as monotherapy. CONCLUSION Thus, treatment of mesothelioma with a combination of gemcitabine with immune checkpoint inhibitors is feasible and results in synergistic clinical response compared to single treatment in the absence of steroids.

DOI: https://doi.org/10.1158/1078-0432.CCR-18-1231

Gemcitabine synergizes with immune checkpoint inhibitors and overcomes resistance in a preclinical model and mesothelioma patients

Paulino Tallón de Lara[1], Virginia Cecconi[1], Stefanie Hiltbrunner[1], Hideo Yagita[2], Martina Friess[3], Beata Bode[4], Isabelle Opitz[3], Bart Vrugt[4], Walter Weder[3], Paul Stolzmann[5], Emanuela Felley-Bosco[3], Rolf A. Stahel[6], Verena Tischler[4], Christian Britschgi[6], Davide Soldini[4], Maries van den Broek[1]* and Alessandra Curioni-Fontecedro[6]*

[1]Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland;

[2]Department of Immunology, Juntendo University School of Medicine, Tokyo, 113-8421, Japan;

[3]Department of Thoracic Surgery, University Hospital Zurich, Zurich, Switzerland;

[4]Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland;

[5]Department of Nuclear Medicine, University Hospital Zurich, Zurich, Switzerland;

[6]Department of Hematology and Oncology, University Hospital Zurich, Zurich, Switzerland

*These authors share the last and corresponding authorship. Maries van den Broek, Institute of Experimental Immunology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. vandenbroek@immunology.uzh.ch. Alessandra Curioni-Fontecedro, Department of Hematology and Oncology, Division of Oncology, University Hospital Zurich, Raemistrasse 100, 8091 Zurich, Switzerland. alessandra.curioni@usz.ch.

The authors declare no potential conflicts of interest.

Running title: Gemcitabine synergizes with immune-checkpoint inhibitors

Abstract

Purpose: Combination of immune checkpoint inhibitors with chemotherapy is under investigation for cancer treatment.

Experimental design: We studied the rationale of such a combination for treating mesothelioma, a disease with limited treatment options.

Results: The combination of gemcitabine and immune checkpoint inhibitors outperformed immunotherapy alone with regard to tumor control and survival in a pre-clinical mesothelioma model; however, the addition of dexamethasone to gemcitabine and immune checkpoint inhibitors nullified the synergistic clinical response. Further, treatment with gemcitabine plus anti-PD-1 resulted in an objective clinical response in two mesothelioma patients, who were resistant to gemcitabine or anti-PD-1 as monotherapy.

Conclusion: Thus, treatment of mesothelioma with a combination of gemcitabine with immune checkpoint inhibitors is feasible and results in synergistic clinical response compared to single treatment in the absence of steroids.

Statement of significance:

Mesothelioma is an aggressive cancer with few therapeutic options. Although immunotherapy might improve the course of the diseases, we are often confronted with the challenge of resistance. It is therefore of paramount importance to overcome such resistance and to improve patients' outcome. We have demonstrated here the synergy of a chemotherapy (gemcitabine) and immune-checkpoint inhibitors in a preclinical model and in patients. These findings are essential for the design of new therapeutic approaches.

Introduction

T cells can recognize and control transformed cells (1), however, protective immunity against cancer often is insufficient due to a plethora of immune escape mechanisms, including immune checkpoints such as programmed death receptor 1 (PD-1, CD279) (2). PD-1 is expressed on the surface of activated T cells (3) and interaction with its ligands PD-L1 (CD274, B7-H1) or PD-L2 (CD273, B7-DC) (4) results in T cell unresponsiveness (5). PD-L1 can be expressed by T and B cells, myeloid cells, endothelial cells and by various tumors (6), whereas the expression of PD-L2 is limited to dendritic cells (DCs), macrophages and mast cells (4).

Blockade of the interaction between PD-1 and its ligands has shown remarkable clinical responses in a proportion of patients with melanoma or non-small cell lung cancer (NSCLC) (7, 8). Also in mesothelioma, treatment with immune checkpoint inhibitors (ICI) is promising with responses in about 20% (9) and disease control rate in 50% of patients (presented at ESMO 2017, Zalckman G., abstract LBA58_PR). Indeed, the efficacy of ICI in mesothelioma has been shown in different studies (9, 10), It is still a matter of debate whether the expression of PD-L1 by the tumor cells might be a useful predictive marker for survival or for clinical responsiveness to treatment with anti-PD-L1 (11-13). These discordant data may be explained by the difficulty to faithfully stain tumor samples for PD-L1, as well as the availability of multiple staining protocols, which hampered conclusive interpretations regarding the prognostic relevance of PD-L1 expression in many cancer types including mesothelioma (14, 15).

In this study, we show that the expression of PD-L1 by mesothelioma cells has prognostic power in a large cohort of mesothelioma patients. Furthermore, we demonstrate feasibility and efficacy of combining gemcitabine with ICI in a preclinical model for mesothelioma. We chose to combine gemcitabine with ICI because of its common use in mesothelioma and immune-

stimulating effects (16-18). Finally, we tested this combination in two patients, who did not respond to pembrolizumab or gemcitabine as monotherapy, and observed clinical response in both cases.

Materials and Methods

Clinico-pathological characteristics of human mesothelioma samples

Large sections from 145 mesothelioma patients were collected between 1999 and 2009. Patients received either a combination treatment with cisplatin/gemcitabine (with cisplatin on day 1 and gemcitabine on day 1 and 8, every three weeks) or cisplatin/pemetrexed (both on day 1, every three weeks). Post-chemotherapy tumor samples were collected after three cycles of treatment. In total, we collected 251 samples of which 147 were of the epithelioid, 14 of the biphasic and 90 of the sarcomatoid subtype. The 251 samples were collected as follows: 117 were surgical biopsies, 132 were surgical specimens and 2 were needle-core biopsies. From those 251 samples, 118 were collected before and 133 after chemotherapy; 160 of these samples were matched samples from 80 patients, taken before as well as after chemotherapy. From this cohort of 145 patients, matching samples of 80 patients before and after chemotherapy were available. Clinical data are summarized in Supplementary Table S1. All patients gave informed, written consent and the study was conducted in accordance with the declaration of Helsinki. The study was approved by the Institutional Ethical Review Board of the University Hospital Zurich under reference number StV 29-2009 and EK-ZH 2012-0094.

Human mesothelioma tissue microarray

Formalin-fixed paraffin-embedded (FFPE) blocks of the abovementioned samples were retrieved

from the archives of the Institute of Surgical Pathology, University Hospital of Zurich. All cases were reviewed by two pathologists (BV and VT) on full sections of a representative tumor block and classified following the definitions of the WHO classification for each subtype of mesothelioma. In case of biphasic mesothelioma, areas were identified that clearly separated the epitheloid and sarcomatoid growth patterns and 2 cores were taken from each area.

Tissue microarrays (TMAs) were constructed with a custom-made, semiautomatic tissue array (Beecher Instruments, Sun Prairie, WI) as previously described (19, 20). The TMAs were built using at least two tissue punches with cores of 0.6 mm diameter for each patient using a Ventana ES instrument (Ventana Medical Systems, Baar, Switzerland) (21). TMA blocks were sectioned and stained with hematoxylin and eosin for morphologic assessment. Additional cores of control tissues, including 8 tonsils, 8 normal lung tissues, 8 normal pleura and 8 adenocarcinomas of the lung, were included in duplicate. Technical factors leading to loss of cores during TMAs processing (i.e., sectioning and staining) resulted in a reduction of the patient numbers available for subsequent statistical analysis.

Human mesothelioma whole tumor sections

In addition, we analyzed 20 matching whole tumor sections from 10 patients, taken before and after chemotherapy. These samples are included in the TMA described above. Those patients were treated with cisplatin (75 mg/m2 Q3W) plus pemetrexed (500 mg/m2 Q3W) as part of a clinical trial (22).

Human mesothelioma cell lines and *in vitro* treatment

The following human mesothelioma cell lines were used in this study: ZL55 (epitheloid), SPC111 (sarcomatoid) and MSTO-211H (biphasic). ZL55 and SPC111 were generated in our

laboratory (23) and MSTO-211H (24) was obtained from ATCC. Cells were cultured in humidified incubator at 37°C and 5% CO2 in either DMEM (for ZL55 and SPC11) or RPMI (for MSTO-211H) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics (all from Gibco). Cells were tested negative for *Mycoplasma* ssp. using the VenorGeM Mycoplasma detection kit (Sigma).

Gemcitabine (Eli Lilly and Company), cisplatin (Sigma Aldrich) and pemetrexed (Eli Lilly and Company) were obtained from the local pharmacy and dissolved in phosphate-buffered saline (PBS). Final concentrations in cell culture were as follows: gemcitabine, 0.1, 0.2, 0.5, 1 and 10 µM; cisplatin, 0.01, 0.1 and 1 µM; pemetrexed, 0.1, 1 and 10 µM. The drug was added at the start of culture and once more after 24 h. Media was used as negative control (25). Analysis was performed 48 h after start of cultures.

Mouse experiments

C57BL/B6 mice were obtained from Harlan Laboratories (Envigo) and kept under specific pathogen-free conditions at the Laboratory Animal Services Center (LASC) of the University of Zurich. Six to eight weeks old female mice were used for all experiments. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by the Cantonal Veterinary Office Zurich.

RN5 cells are crocidolite-induced mesothelioma cells of C57BL/6 origin (26) (27). RN5 cells were tested negative for *Mycoplasma* ssp. using the VenorGeM Mycoplasma detection kit (Sigma). C57BL/6 mice were injected subcutaneously (s.c.) with 10^6 RN5 cells in 100 µl phosphate-buffered saline (PBS). Tumor growth was measured every 3-4 days in two dimensions (length and width) with a caliper. When tumors reached a size of 40-50 mm^2 (approximately 40 d after tumor cell injection), mice were randomized into different treatment groups and treatment

was started. The death event was defined as tumor size reaching the legally acceptable limit of 225 mm^2.

Gemcitabine (Eli Lilly and Company) was dissolved in PBS and given intraperitoneally (i.p.) once per week at a dose of 120 mg/g body weight (28). Anti–CTLA-4 (clone UC10-4F10-11) and anti-PD-1 (clone RMPI-14) were purified from culture supernatant using protein G Sepharose 4 Fast Flow (GE Healthcare) columns according to the manufacturer's protocol. Antibodies were administered i.p. once per week at 250 μg/100 μl PBS (29). Dexamethasone (Sigma-Aldrich) was administered orally (p.o.) once per week at 0.3 mg/kg body weight (30). All treatments were given on the same day. This experiment was repeated two times with at least 5 mice/group.

Flow cytometry and immunohistochemistry on cell lines

Cultured cells were harvested by 0.5% Trypsin-EDTA treatment and stained with the following fluorophore-labeled monoclonal antibodies and appropriate isotype controls: PD-L1 (clone 29E.2A3), PD-L2 (clone 24F.10C12), HLA-ABC (clone G46- 2.6), HLA-DR (clone L243). Dead cells were stained using Zombie Violet Fixable Viability Kit. Anti-HLA-ABC and anti-HLA-DR were obtained from BD Biosciences, all other reagents from BioLegend. Cells were incubated with appropriately diluted antibodies in PBS for 25 minutes at 4°C. Subsequently, cells were washed and fixed for 5 minutes with 2% paraformaldehyde in PBS. Samples were measured on a CyAn ADP9 machine (Beckman Coulter) and analyzed using FlowJo v9.8.5 software (Tree Star). For IHC, cell lines were pelleted after in vitro treatment, and formalin-fixed, paraffin-embedded, sectioned and subjected to immunohistochemistry for PD-L1 using clone E1L3N as described below under “Immunohistochemistry”.

Human patients treated with combination of gemcitabine with anti-PD-1

The use of gemcitabine plus pembrolizumab was justified by the fact that both drugs are approved as monotherapy. The combination treatment was used off-label with written informed consent of the patients according to the declaration of Helsinki and was approved by the University Hospital Zurich.

Immunohistochemistry

Human samples

For immunohistochemistry (IHC), 4 µm thick paraffin sections were cut from the TMA block and mounted on silane-coated glass slides. The sections were stained using an automated IHC platform (Ventana Benchmark). The following antibodies were used: anti-PD-L1 (E1L3N, Cell Signaling), anti-FOXP3 (236A/E7, Abcam), anti-CD3 (clone LN10, Leica Biosystems), anti-Perforin (clone 5B10, DBS). Anti-PD-L1 staining with E1L3N antibody was performed by pretreatment of samples in H_2O_2 for 30 min and then samples were incubated with the antibody diluted as 1:1000 at room temperature for 30 min and heat-induced epitope retrieval HIER2 solution with a Bond Polymer Refine Detection kit (Leica Biosystems). Samples with <50 evaluable cells were excluded. Biopsies were considered PD-L1$^+$ when >1% of tumor cells were stained positive with anti-PD-L1. Comparison of PD-L1 staining using either the clone E1L3N from Cell Signaling or the clone SP142 from Ventana showed a correspondence in 100% of cases either negative or positive (Supplementary Figure S1). Each core was evaluated independently by two pathologists (DS and VT). We used archived FFPE samples. Although the use of archival FFPE samples to determine PD-L1 expression in mesothelioma has not yet been validated, it was shown that matching fresh and FFPE lung cancer samples gave similar results regarding PD-L1 expression determined by IHC (31).

Murine samples

Resected tumors were processed for FFPE and 4 µm thick sections were stained for IHC by Sophistolab AG (Muttenz, Switzerland) using a Leica BondMax instrument and Refine HRP-Kits (Leica DS9800, Leica Microsystems Newcastle) according to the manufacturer's guidelines. The following antibodies were used: PD-L1 (Lifespan LS-B9795), FoxP3 (Novus Biologicals, NBP1-18319), CD3 (RMAB005, clone SP7, DBiosystem). Hematoxylin counterstaining was performed according to standard protocol. Whole slides were scanned with a Zeiss Mirax Midi slide scanner (20x objective, NA0.8) equipped with a 3-CCD color camera (Hitachi HV-F22) and analyzed using Pannoramic viewer 1.15.4 (3DHISTECH).

Survival Analysis

The Kaplan-Meier method was used to estimate tumor-specific overall survival (OS). Tumor-specific OS in humans was determined from the date of histological diagnosis of mesothelioma to the date of death from the same cancer up to 5 years of follow-up. Patients, who were alive at the time of last follow-up were censored for OS analysis. Continuous data were compared between groups using the Mann-Whitney U test. For comparison of experimental groups, two-tailed Student's *t*-test with Welch's correction was performed. Statistical significance $p<0.05$. Statistical analyses were performed using IBM SPSS Statistics 20.0 (SPSS Inc, Chicago IL)

For mice, tumor-specific OS was determined from the date of randomization and the death event was defined as tumor size reaching the legally acceptable limit of 225 mm^2. For survival analysis, the Log-rank Test (Mantel Cox test) was used. For comparison between each experimental groups ANOVA with Bonferroni correction was performed. Statistical analyses were performed using PRISM (GraphPad Prism version 7 for Mac, GraphPad Software, La Jolla California USA).

Results

Expression of PD-L1 by human mesothelioma is a negative prognostic biomarker for survival

We investigated the expression of PD-L1 in a cohort of 145 mesothelioma patients (Supplementary Table S1). Tumor samples were available from 100 cases before chemotherapy and 122 cases after chemotherapy; 80 of these cases were paired samples taken from the same patient before and after chemotherapy. We detected expression of PD-L1 in 14% of samples before chemotherapy (14 out of 100) and in 18% of cases after chemotherapy (22 out of 122) (Supplementary Table S2); expression of PD-L1 was membranous and homogenous, and the intensity of expression varied from low (+) to high (+++) (Figures 1A and 1B). We found that expression of PD-L1 on tumor cells was a negative prognostic marker (p=0.04) for survival independent of both the histological subtype and other clinical features (Figure 1C).

Using large sections, we detected $CD3^+$ T cells throughout the tumor tissue, but none of them expressed perforin. Instead, most of the $CD3^+$ T cells expressed $FOXP3^+$ and were preferentially localized in PD-$L1^+$ tumor areas (Supplementary Figure S2).

Chemotherapy does not influence PD-L1 expression by human mesothelioma

To investigate whether chemotherapy with cisplatin/pemetrexed or cisplatin/gemcitabine has an impact on the expression of PD-L1 by mesothelioma cells, we compared the expression of PD-L1 in paired samples taken before and after chemotherapy from 80 patients. Although we found that the PD-L1 expression differed between matched samples, these changes seemed random (Supplementary Table S2), suggesting that the chemotherapies investigated here had no straightforward impact on PD-L1 expression. From the cohort of 80 paired samples taken before

and after chemotherapy, we further analyzed 20 large sections (i.e. paired samples from 10 patients) for expression of PD-L1 on tumor cells and confirmed the data described above (not shown).

To directly address whether chemotherapeutic drugs influence the expression of PD-L1 on mesothelioma cells, we incubated two human PD-L1^{+} mesothelioma cell lines (MSTO and SPC11) and one human PD-L1^{-} mesothelioma cell line (ZL55) with gemcitabine or cisplatin/pemetrexed. We did not observe chemotherapy-induced changes with respect to PD-L1 expression by FACS in any of the cell lines (Figure 2). To further validate our data, we made cell blocks of the previously mentioned conditions and perform IHC for PD-L1 (Supplementary Figure S3). There was a complete correlation between FACS and IHC for MSTO (positive) and ZTL55 (negative) cell lines (either untreated, treated with gemcitabine or treated with cisplatin/pemetrexed). The SPC11 cell line (positive by FACS) was weakly positive only in one out of the three samples (untreated, treated with gemcitabine or treated with cisplatin/pemetrexed). This is consistent with reports showing variation among different PD-L1 antibodies (32) and that PD-L1 analysis by FACS and IHC is not fully overlapping (33).

Together these data suggest that neither cisplatin/pemetrexed nor cisplatin/gemcitabine induce direct changes in PD-L1 expression by mesothelioma cells. Rather, we think that the random differences observed between paired samples may be explained by intratumoral heterogeneity regarding PD-L1 expression or by indirect effects of chemotherapy, For example, some chemotherapies including cisplatin and doxorubicin induce immunogenic cell death and result in local stimulation of tumor-specific immunity (34) and this will produce interferon-γ (IFN-γ) at the tumor site. PD-L1 expression over time could be then the result of local IFN-γ production (35). Furthermore, our data suggest that standard of care chemotherapy does not *per se* preclude subsequent treatment with PD-1 blocking antibodies.

Gemcitabine plus immune checkpoint has better efficacy than gemcitabine or immune checkpoint inhibitors as monotherapy in mice

To investigate whether gemcitabine synergizes with ICI, we used a mouse mesothelioma model, subcutaneous RN5 tumors in syngeneic C57BL/6 mice. This model shares histopathologic features with the aggressive human mesothelioma subtype (sarcomatoid). We found that treatment with gemcitabine impeded tumor progression and promoted infiltration by $CD3^+$ T cells, whereas treatment with ICI had no significant impact on tumor progression or survival. The combination of gemcitabine and ICI, however, significantly prolonged survival and resulted in tumor rejection in a proportion of the mice (Figures 3A and 3B), suggesting a synergistic effect of ICI plus gemcitabine.

Corticosteroids nullify the synergy of chemo- and immunotherapy in mice

Dexamethasone is an anti-inflammatory and immunosuppressive glucocorticoid that is commonly used together with chemotherapy as an anti-emetic drug. Specifically, dexamethasone is often given with gemcitabine in order to prevent side effects including chemotherapy-induced nausea and vomiting, fever and chills. In contrast to brief treatment schedules, long-term use of glucocorticoids is immunosuppressive (24). We observed a therapeutic synergy when ICI was combined with gemcitabine, suggesting that activation of immune defense supports the clinical response to gemcitabine. Therefore, we wondered whether long term treatment with dexamethasone would negatively influence this therapeutic synergy and treated mice with established RN5 tumors with gemcitabine plus ICI or gemcitabine plus ICI plus dexamethasone. Mice treated with gemcitabine alone or vehicle served as controls. Again, we observed that gemcitabine plus ICI resulted in significantly better survival compared to gemcitabine alone

(Figure 3B). However, addition of dexamethasone to the treatment with gemcitabine plus ICI significantly reduced responses in treated mice (Figure 3B). Next, we investigated the immune infiltrate in mouse mesothelioma by immunohistochemistry for CD3 and FoxP3 at the endpoint. We observed increased fibrotic and necrotic areas after treatment with gemcitabine plus ICI, as well as increased number of infiltrating $CD3^+$ T cells compared to all other groups (Figure 4). Furthermore, none of the $CD3^+$ cells expressed FoxP3 (data not shown). In contrast, tumors of mice treated with gemcitabine plus ICI plus dexamethasone did not show necrotic areas and very few infiltrating $CD3^+$ T cells (Figure 4), suggesting that corticosteroids negatively influence the clinical response to gemcitabine plus ICI. We think that this may apply to any other therapy that involves immune stimulation including standard therapies (36) (37), suggesting that the use of steroids in the context of cancer therapies should be avoided if possible. It may very well be of general applicability that clinical responses to ICI are stronger in absence of steroids. The finding that concomitant use of steroids was associated with poor outcomes of NSCLC patients treated with ICI (38) supports this idea.

The combination of gemcitabine with anti-PD-1 can overcome the resistance to ICI in patients

Currently, the standard of treatment of mesothelioma is chemotherapy. However, blockade of the interaction between PD-1 and its ligands has shown promising clinical responses in a proportion of patients in clinical trials (9). We present here two patients with mesothelioma who were refractory to various therapies but responded to a combination of anti-PD-1 and gemcitabine.

The first patient was 66 years old and diagnosed with epithelioid mesothelioma three years ago (in 2014). The patient underwent multimodality approaches including chemotherapy and tumor resection. Due to relapse of disease, the patient was treated with pembrolizumab. Upon

progression, the patient was treated with carboplatin/pemetrexed. Then he was switched to carboplatin/gemcitabine and consecutively to gemcitabine monotherapy. Due to further progression, the patient received a combination of gemcitabine (1000 mg/m^2 weekly) plus pembrolizumab (200 mg every 3 weeks) without corticosteroids. Before starting this treatment, the patient complained about dysphagia and weight loss. However, two months after starting this combination treatment, the patient could eat normally and a radiological and metabolic response to treatment was detected (Figures 5A and 5B). He is still under therapy, 20 weeks after start of treatment.

The second case was a 57-year-old patient diagnosed with epithelioid mesothelioma 4 years ago (in 2013). The patient underwent multimodality approaches including chemotherapy and tumor resection (Figure 5C). Due to recurrence of disease in 2016, the patient was treated with pembrolizumab (anti-PD-1), which resulted in progression of disease (Figure 5D, time point i), and subsequently with carboplatin/pemetrexed with further progression of disease (Figure 5D, time point ii). Then one symptomatic lesion in the chest wall was irradiated with a total dose of 36 Gy (6 x 6 Gy). Restaging was performed (Figure 5D, time point iii) before start of weekly chemotherapy with gemcitabine (without corticosteroids). Within two months of gemcitabine monotherapy there was evidence of progression of the mediastinal mass and lung metastasis (Figure 5D, time point iv) and the patient had a reduced performance status with ECOG 3 due to cachexia and dyspnea. Because the patient did not show any limiting side effects on gemcitabine, we started a treatment with pembrolizumab (200 mg every three weeks) and gemcitabine (1000 mg/m^2 weekly), in March 2017. After three cycles of this treatment, the patient clinically improved and went back to work using his bicycle as means of transport. Furthermore, radiological images showed a clinical response of the mediastinal mass and lung metastasis with increased pleural effusion (Figure 5D, time point v). We observed no side effects besides grade 2

anemia and pleural effusion that was drained after treatment. We analyzed the composition of the pleural effusion and observed that together with few mesothelioma cells there was 80% of lymphocytes (Figures 6A and 6B), of these, 90% were $CD3^+$ T cells, consisting of 20% $CD4^+$ (Figure 6C) and 75% $CD8^+$ cells (Figure 6D). This suggests that the combination of gemcitabine plus anti-PD-1 mobilized protective immunity resulting in an objective clinical response. Because of his improved general condition, the patient wished to stop the treatment and undertake a journey of five weeks. Upon his return, treatment with gemcitabine plus anti-PD-1 was started with a three-weekly schedule for both medications as per the patient's request. After two cycles of this treatment, a progression of disease was detected and the treatment was discontinued.

Discussion

Combining different therapies for cancer is a promising approach to overcome resistance and improve responses. For example, combination of nivolumab (anti-PD-1) with ipilimumab (anti-CTLA-4) has shown improved clinical responses compared to monotherapy, however, also increased side effects (39). Currently, combinations of different chemotherapies with immunotherapy are under evaluation for different tumor types (NCT02039674, NCT02366143). We investigated whether the combination of gemcitabine with ICI is an effective and feasible treatment for mesothelioma that outperforms monotherapy and may even overcome resistance to monotherapy.

Expression of PD-L1 by tumor cells is often used as inclusion criterion for treatment with antibodies that block the interaction of PD-1 with its ligands (9). Therefore, we first investigated whether gemcitabine influences the expression of PD-L1 by mesothelioma cells *in vivo* and *in vitro*. In addition, we found that the expression of PD-L1 is a negative biomarker for survival in

mesothelioma, which goes in line with previous studies (40) (14) and with data obtained from other tumor entities (41) (42) (43) (44).

We found that treatment with gemcitabine plus ICI significantly prolonged survival of mesothelioma-bearing mice compared to gemcitabine alone, whereas ICI as monotherapy had no impact. Although ICI as monotherapy shows clinical efficacy in mesothelioma patients (45), this is not the case in our preclinical model. Absence of clinical efficacy of ICI as monotherapy has been described in multiple mouse models (29) but this discrepancy with humans is not understood.

Corticosteroids are commonly used to manage side effects of chemotherapy. Because of their immunosuppressive properties, we evaluated the impact of dexamethasone on the clinical efficacy of gemcitabine plus ICI and observed an abrogation of therapeutic synergy. This suggests that side effects of chemotherapy should be managed by other medications than corticosteroids, whenever possible. Indeed, we have successfully applied this approach to both patients presented here.

Our data show that PD-L1 expression is heterogeneous and prone to immune response-induced changes. In addition, various studies have shown a high degree of intratumoral heterogeneity in PD-L1 expression in different cancers (46) (47). Therefore, we think that using PD-L1 expression as an inclusion criterion for treatments targeting this pathway is not sufficiently justified for mesothelioma patients. We demonstrated the synergy of gemcitabine with ICI providing a rationale for a clinical trial that can include patients with different tumor types, where gemcitabine represents a standard of care and might be favorable in terms of tolerability and costs compared to combination of several immunomodulating agents. It has been shown that in patients with advanced lung cancer that daily administration of steroids reduces lymphocyte numbers (48) and may be associated with worse outcome (38). Obviously, highly emetogenic treatments must

be combined with anti-emetic drugs, however, corticosteroids should be avoided and established alternatives used instead. Here, we show in a pre-clinical model that avoiding the use of dexamethasone improves the efficacy of immunotherapy for mesothelioma, a disease with very limited effective treatment options (49) and an unmet need for new therapies (50).

Acknowledgements

This work was financially supported by the Sophien Stiftung Zurich, the Swiss National Science Foundation, the University of Zurich (University Research Priority Project "Translational Cancer Research"), the Forschungskredit of the University of Zurich and the Science Foundation for Oncology (SFO).

We thank André Fitsche, Susanne Dettwiler and Martina Storz (Institute for Surgical Pathology, University Hospital Zurich) for excellent technical assistance with TMA construction and establishment of IHC staining. We thank the personnel of the Laboratory Animal Services Center (LASC) at the University of Zurich for expert animal care.

Figure legends

Figure 1. PD-L1 is a negative prognostic marker for mesothelioma patients.

PD-L1 Expression in mesothelioma tumor samples. (A) Representative example of negative expression. (B) Representative example of positive expression (brown). The expression of PD-L1 was homogeneous within a core of the TMA. (C) Overall survival data for patients with PD-L1-positive versus -negative mesothelioma. The PD-L1 negative patients had a median survival of 20.7 months (95% CI, 16.8-24.5 months), the PD-L1 positive patients of 8.8 months (95% CI, 0.9-16.6 months). Continuous data were compared between groups using the Mann-Whitney U test.

Figure 2. Chemotherapy does not change the expression of PD-L1 on human mesothelioma cell lines *in vitro*.

MSTO, ZL55 and SPC11 cells were treated *in vitro* with 0.2 µM gemcitabine or 0.1 µM cisplatin/1 µM pemetrexed for 48 h. Surface PD-L1 expression was determined by flow cytometry. Cells cultured in media served as control. PD-L1 expression on untreated cells is displayed in light red, after chemotherapy in dark red. Staining with isotype controls of untreated and chemotherapy-treated cells is displayed in light and dark grey, respectively.

Fig 3. Gemcitabine plus ICI significantly prolongs the survival of mesothelioma-bearing mice. Female C57BL/6 mice were injected s.c. with 10^6 syngeneic RN5 mesothelioma cells. Mice were randomized into treatment groups as soon as tumors reached a size of 40-50 mm^2 (appr. 40 d after tumor cell injection). Mice were treated as follows: (A) Untreated (n=4); Gemcitabine (Gem, n=5); anti-CTLA4 + anti-PD1 (ICI, n=5); Gem + ICI (n=4), or (B) Untreated

(n=6); Gem (n=6); Gem + ICI (n=7); Gem + ICI + Dexa (Dexamethasone, n=7). Statistical analysis was performed using a Log-rank test (Mantel-Cox test). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The experiments depicted in panel A and B were performed independently and represent one experiment repeated two times.

Figure 4. **Gemcitabine plus immune checkpoint inhibitors synergize to induce tumor cell death and infiltration by CD3$^+$ cells.**

Representative immunohistochemical staining of CD3$^+$ cells in tumor samples from mice described in Figure 4B.

Fig 5. Response to pembrolizumab and gemcitabine in two patients with mesothelioma.

Panels A-B, patient 1; panels C-D, patient 2. (A) Timeline of the treatments. (B) Quantification of the response to the treatments by PET/CT scan at 5 different timepoints (1-5), black arrow marks the start of gemcitabine + pembrolizumab therapy. Performance status was evaluated by Eastern Cooperative Oncology Group (ECOG) scale. MTV stands for metabolic tumor volume. Lower panel shows representative pictures of whole body PET/CT scan at the corresponding 5 timepoints. (C) Timeline of the treatments. (D) Quantification of the response to the treatments by CT scan 5 different time points (i-v), black arrow marks the start of gemcitabine + pembrolizumab therapy. Performance status was evaluated by Eastern Cooperative Oncology Group (ECOG) scale. Lower panel shows representative pictures of the CT scan of the mediastinum at the corresponding 5 time points. Mediastinal lesion is marked with a white asterisk. White arrow marks the pleural effusion. Lung metastasis in the left upper lower that appeared after radiotherapy (time point iii) is shown in the inlet, marked with a black circle.

Figure 6. Cytological analysis of the pleural fluid from patient 2.

Pleural fluid of patient 2 was collected from the lesion shown in Figure 6.2 E and the sediment was analyzed by cytology. **(A)** Papanicolooau staining revealed the presence of mesothelioma cells (black arrows), numerous lymphocytes (red arrows) and few macrophages and neutrophilic granulocytes in the background (original magnification 400x). Mesothelioma cells were calretinin^{+}, desmin^{-}, BAP1^{-} (data not shown). **(B)** CD3 immunostaining revealed that 90% of lymphocytes were CD3^{+} T-cells (brown signal; original magnification 400x). **(C, D)** Flow cytometric analysis showed that 20% of CD3^{+} T-cells express CD4 (panel C) and 75% express CD8 (panel D).

References

1. Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res. 1970;13:1-27.

2. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol. 2007;8(3):239-45.

3. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol. 2004;4(5):336-47.

4. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol. 2001;2(3):261-8.

5. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol. 2005;6(3):280-6.

6. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med. 2000;192(7):1027-34.

7. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366(26):2455-65.

8. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443-54.

9. Alley EW, Lopez J, Santoro A, Morosky A, Saraf S, Piperdi B, et al. Clinical safety and activity of pembrolizumab in patients with malignant pleural mesothelioma (KEYNOTE-028): preliminary results from a non-randomised, open-label, phase 1b trial. Lancet Oncol.

2017;18(5):623-30.

10. Quispel-Janssen J, van der Noort V, de Vries JF, Zimmerman M, Lalezari F, Thunnissen E, et al. PD-1 blockade with nivolumab in patients with recurrent Malignant Pleural Mesothelioma. J Thorac Oncol. 2018.

11. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature. 2014;515(7528):563-7.

12. Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. Clin Cancer Res. 2009;15(20):6341-7.

13. Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res. 2006;66(7):3381-5.

14. Mansfield AS, Roden AC, Peikert T, Sheinin YM, Harrington SM, Krco CJ, et al. B7-H1 expression in malignant pleural mesothelioma is associated with sarcomatoid histology and poor prognosis. J Thorac Oncol. 2014;9(7):1036-40.

15. Cedres S, Ponce-Aix S, Zugazagoitia J, Sansano I, Enguita A, Navarro-Mendivil A, et al. Analysis of expression of programmed cell death 1 ligand 1 (PD-L1) in malignant pleural mesothelioma (MPM). PLoS One. 2015;10(3):e0121071.

16. Homma Y, Taniguchi K, Nakazawa M, Matsuyama R, Mori R, Takeda K, et al. Changes in the immune cell population and cell proliferation in peripheral blood after gemcitabine-based chemotherapy for pancreatic cancer. Clinical & Translational Oncology. 2014;16(3):330-5.

17. Duffy AG, Greten TF. Immunological off-target effects of standard treatments in gastrointestinal cancers. Ann Oncol. 2014;25(1):24-32.

18. Rettig L, Seidenberg S, Parvanova I, Samaras P, Curioni A, Knuth A, et al. Gemcitabine depletes regulatory T-cells in human and mice and enhances triggering of vaccine-specific cytotoxic T-cells (vol 129, pg 832, 2011). International Journal of Cancer. 2012;130(7):1714-.

19. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med. 1998;4(7):844-7.

20. Hinterberger M, Reineke T, Storz M, Weder W, Vogt P, Moch H. D2-40 and calretinin - a tissue microarray analysis of 341 malignant mesotheliomas with emphasis on sarcomatoid differentiation. Mod Pathol. 2007;20(2):248-55.

21. Curioni-Fontecedro A, Nuber N, Mihic-Probst D, Seifert B, Soldini D, Dummer R, et al. Expression of MAGE-C1/CT7 and MAGE-C2/CT10 predicts lymph node metastasis in melanoma patients. PLoS One. 2011;6(6):e21418.

22. Stahel RA, Riesterer O, Xyrafas A, Opitz I, Beyeler M, Ochsenbein A, et al. Neoadjuvant chemotherapy and extrapleural pneumonectomy of malignant pleural mesothelioma with or without hemithoracic radiotherapy (SAKK 17/04): a randomised, international, multicentre phase 2 trial. Lancet Oncol. 2015;16(16):1651-8.

23. Schmitter D, Lauber B, Fagg B, Stahel RA. Hematopoietic growth factors secreted by seven human pleural mesothelioma cell lines: interleukin-6 production as a common feature. Int J Cancer. 1992;51(2):296-301.

24. Phelps RM, Johnson BE, Ihde DC, Gazdar AF, Carbone DP, McClintock PR, et al. NCI-Navy Medical Oncology Branch cell line data base. J Cell Biochem Suppl. 1996;24:32-91.

25. Kitazono-Saitoh M, Takiguchi Y, Kitazono S, Ashinuma H, Kitamura A, Tada Y, et al. Interaction and cross-resistance of cisplatin and pemetrexed in malignant pleural mesothelioma cell lines. Oncol Rep. 2012;28(1):33-40.

26. Blum W, Pecze L, Felley-Bosco E, Worthmuller-Rodriguez J, Wu L, Vrugt B, et al. Establishment of immortalized murine mesothelial cells and a novel mesothelioma cell line. In Vitro Cell Dev Biol Anim. 2015;51(7):714-21.

27. H. Rehrauer LW, W. Blum, L. Pecze, T. Henzi, V. Serre-Beinier, C. Aquino, B. Vrugt, M. de Perrot, B. Schwaller, E. Felley-Bosco. How asbestos drives the tissue towards tumors: YAP activation, macrophage and mesothelial precursor recruitment, RNA editing and somatic mutations. Oncogene 2018.

28. Lesterhuis WJ, Salmons J, Nowak AK, Rozali EN, Khong A, Dick IM, et al. Synergistic effect of CTLA-4 blockade and cancer chemotherapy in the induction of anti-tumor immunity. PLoS One. 2013;8(4):e61895.

29. Bransi A, Salgado OC, Beffinger M, Milo K, Silina K, Yagita H, et al. Rational Combination of Immunotherapies with Clinical Efficacy in Mice with Advanced Cancer. Cancer immunology research. 2015;3(11):1279-88.

30. Surace L, Lysenko V, Fontana AO, Cecconi V, Janssen H, Bicvic A, et al. Complement is a central mediator of radiotherapy-induced tumor-specific immunity and clinical response. Immunity. 2015;42(4):767-77.

31. Herbst RS, Baas P, Kim DW, Felip E, Perez-Gracia JL, Han JY, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet. 2016;387(10027):1540-50.

32. Hendry S, Byrne DJ, Wright GM, Young RJ, Sturrock S, Cooper WA, et al. Comparison of Four PD-L1 Immunohistochemical Assays in Lung Cancer. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer. 2018;13(3):367-76.

33. Chargin A, Morgan R, Sundram U, Shults K, Tsay EL, Ratti N, et al. Quantification of

PD-L1 and PD-1 expression on tumor and immune cells in non-small cell lung cancer (NSCLC) using non-enzymatic tissue dissociation and flow cytometry. Cancer immunology, immunotherapy : CII. 2016;65(11):1317-23.

34. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nature reviews Immunology. 2008;8(1):59-73.

35. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, et al. PD-L1 is a novel direct target of HIF-1alpha, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. The Journal of experimental medicine. 2014;211(5):781-90.

36. Surace L, Lysenko V, Fontana AO, Cecconi V, Janssen H, Bicvic A, et al. Complement Is a Central Mediator of Radiotherapy-Induced Tumor-Specific Immunity and Clinical Response. Immunity. 2015;42(4):767-77.

37. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nature Reviews Immunology. 2008;8(1):59-73.

38. Martinez-Bernal G ML, Auclin E, Ferrara R, Planchard D, Remon J, Lahmar J, Boucher ME, Caramella C, Adam J, Gazzah A, Soria JC, Besse B. Baseline corticosteroids (CS) could be associated with absence of benefit to immune checkpoint inhibitors (ICI) in advanced non-small cell lung cancer (NSCLC) patients. . Ann Oncol, 1323P. 2017.

39. Hodi FS, Chesney J, Pavlick AC, Robert C, Grossmann KF, McDermott DF, et al. Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial. Lancet Oncology. 2016;17(11):1558-68.

40. Nguyen BH, Montgomery R, Fadia M, Wang J, Ali S. PD-L1 expression associated with worse survival outcome in malignant pleural mesothelioma. Asia-Pacific journal of clinical oncology. 2018;14(1):69-73.

41. Wu P, Wu D, Li L, Chai Y, Huang J. PD-L1 and Survival in Solid Tumors: A Meta-Analysis. PLoS One. 2015;10(6):e0131403.

42. Chowdhury S, Veyhl J, Jessa F, Polyakova O, Alenzi A, MacMillan C, et al. Programmed death-ligand 1 overexpression is a prognostic marker for aggressive papillary thyroid cancer and its variants. Oncotarget. 2016;7(22):32318-28.

43. Cierna Z, Mego M, Miskovska V, Machalekova K, Chovanec M, Svetlovska D, et al. Prognostic value of programmed-death-1 receptor (PD-1) and its ligand 1 (PD-L1) in testicular germ cell tumors. Ann Oncol. 2016;27(2):300-5.

44. Katsuya Y, Fujita Y, Horinouchi H, Ohe Y, Watanabe S, Tsuta K. Immunohistochemical status of PD-L1 in thymoma and thymic carcinoma. Lung Cancer. 2015;88(2):154-9.

45. Scherpereel A, Mazieres J, Greillier L, Dô P, Bylicki O, Monnet I, et al. Second- or third-line nivolumab (Nivo) versus nivo plus ipilimumab (Ipi) in malignant pleural mesothelioma (MPM) patients: Results of the IFCT-1501 MAPS2 randomized phase II trial. Journal of Clinical Oncology. 2017;35(18_suppl):LBA8507-LBA.

46. Munari E, Zamboni G, Marconi M, Sommaggio M, Brunelli M, Martignoni G, et al. PD-L1 expression heterogeneity in non-small cell lung cancer: evaluation of small biopsies reliability. Oncotarget. 2017;8(52):90123-31.

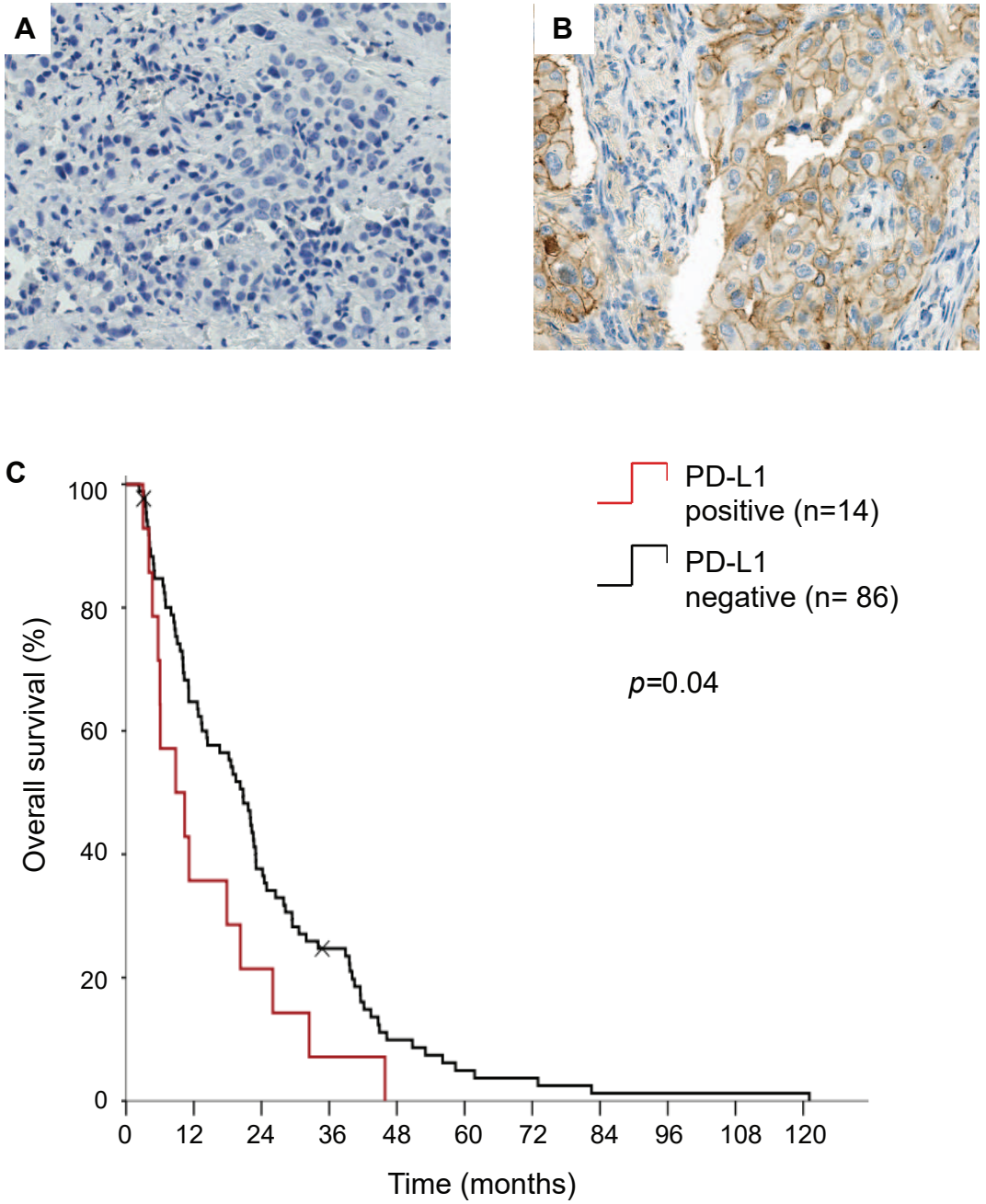
47. Nakamura S, Hayashi K, Imaoka Y, Kitamura Y, Akazawa Y, Tabata K, et al. Intratumoral heterogeneity of programmed cell death ligand-1 expression is common in lung cancer. PLoS One. 2017;12(10):e0186192.

48. Cook AM, McDonnell AM, Lake RA, Nowak AK. Dexamethasone co-medication in cancer patients undergoing chemotherapy causes substantial immunomodulatory effects with implications for chemo-immunotherapy strategies. Oncoimmunology. 2016;5(3):e1066062.

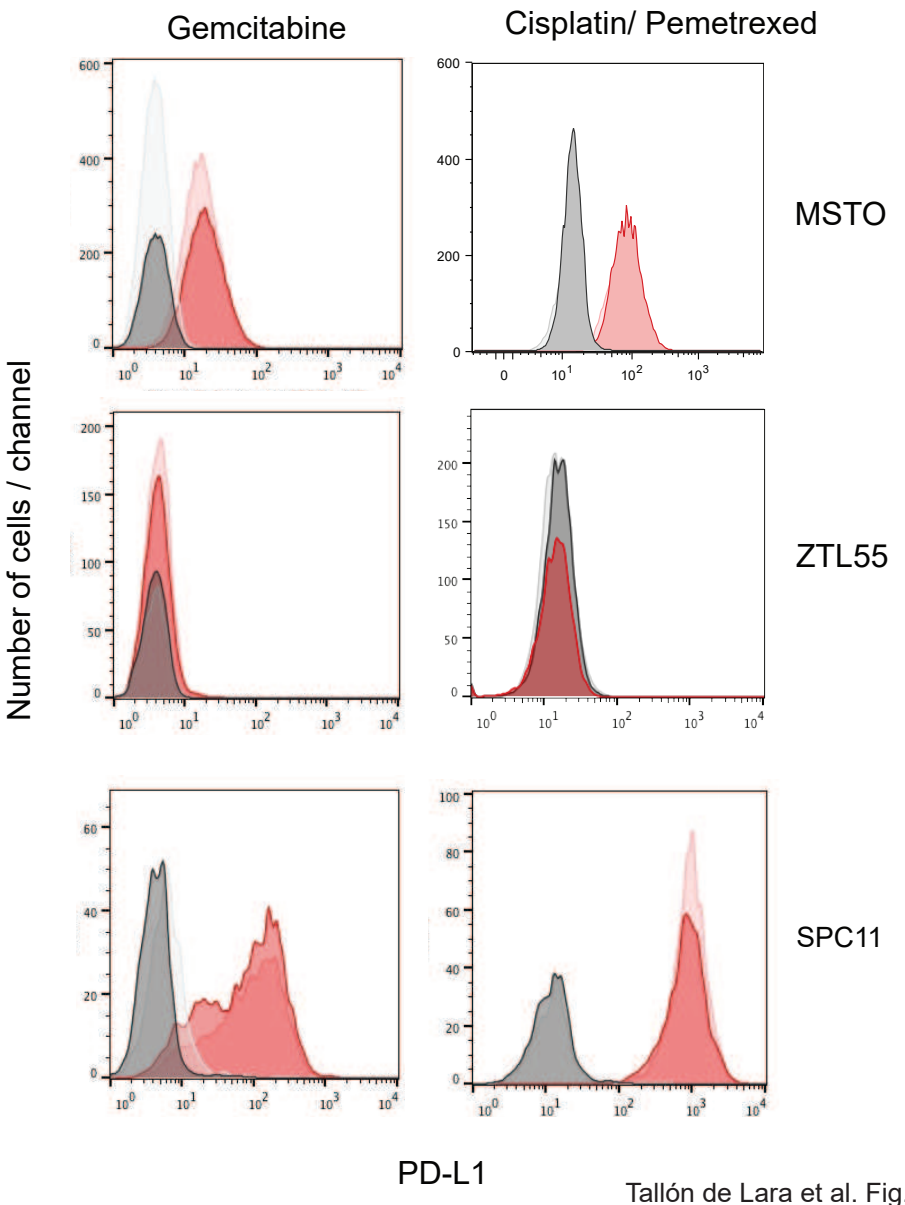
49. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, et al. Phase

III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol. 2003;21(14):2636-44.

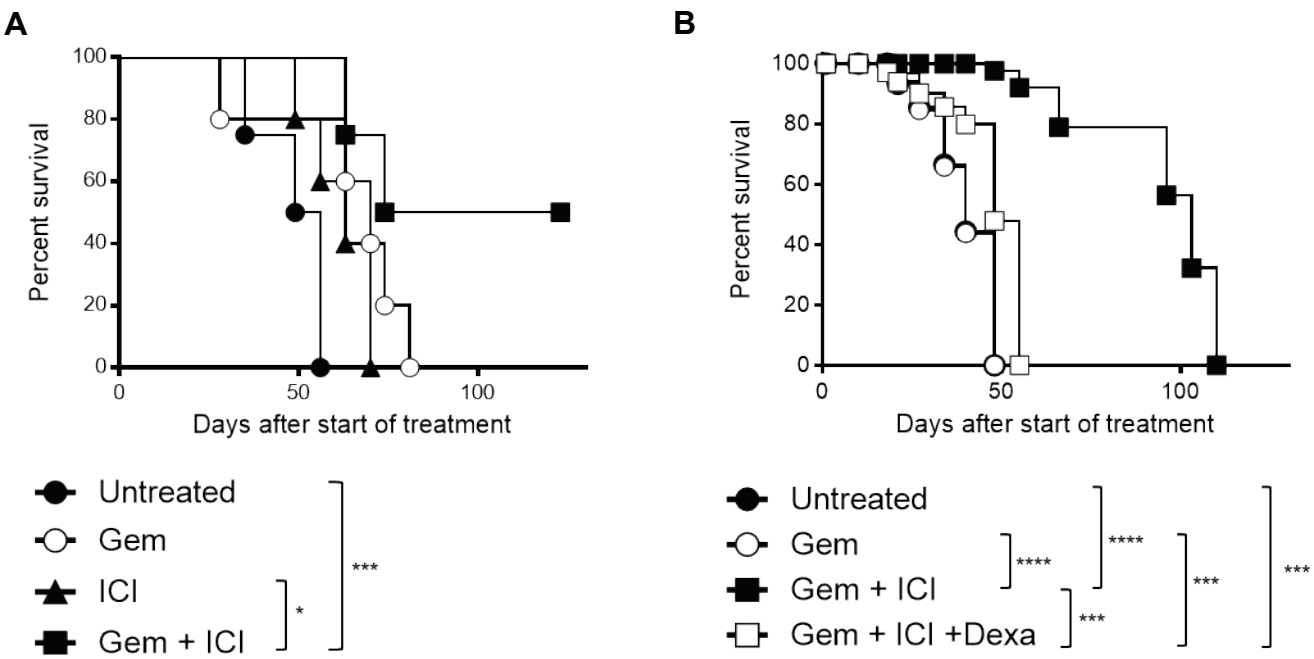
50. Stahel RA, Weder W, Felley-Bosco E, Petrausch U, Curioni-Fontecedro A, Schmitt-Opitz I, et al. Searching for targets for the systemic therapy of mesothelioma. Ann Oncol. 2015;26(8):1649-60.



Tallón de Lara et al. Fig. 1

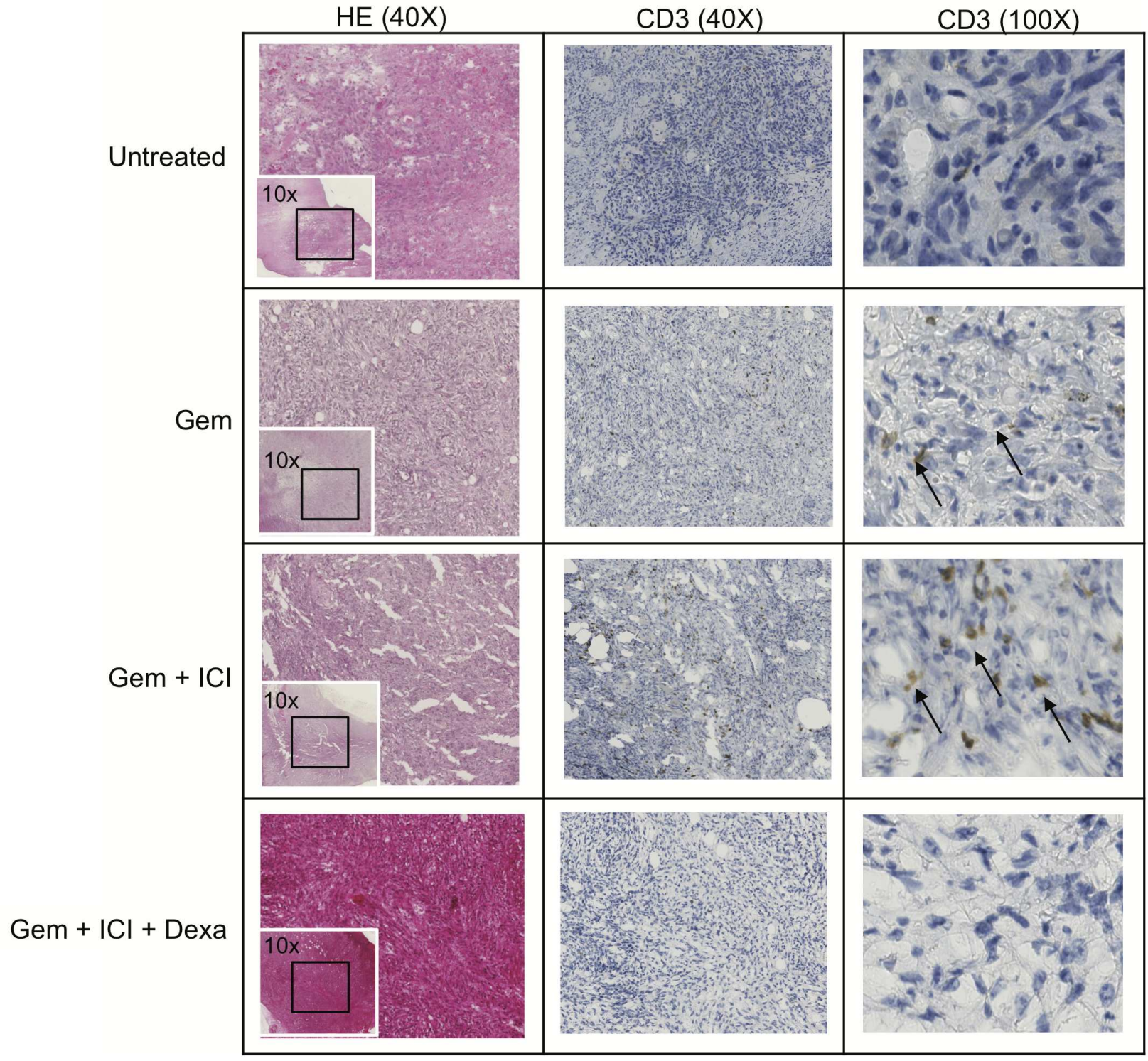


Tallón de Lara et al. Fig. 2

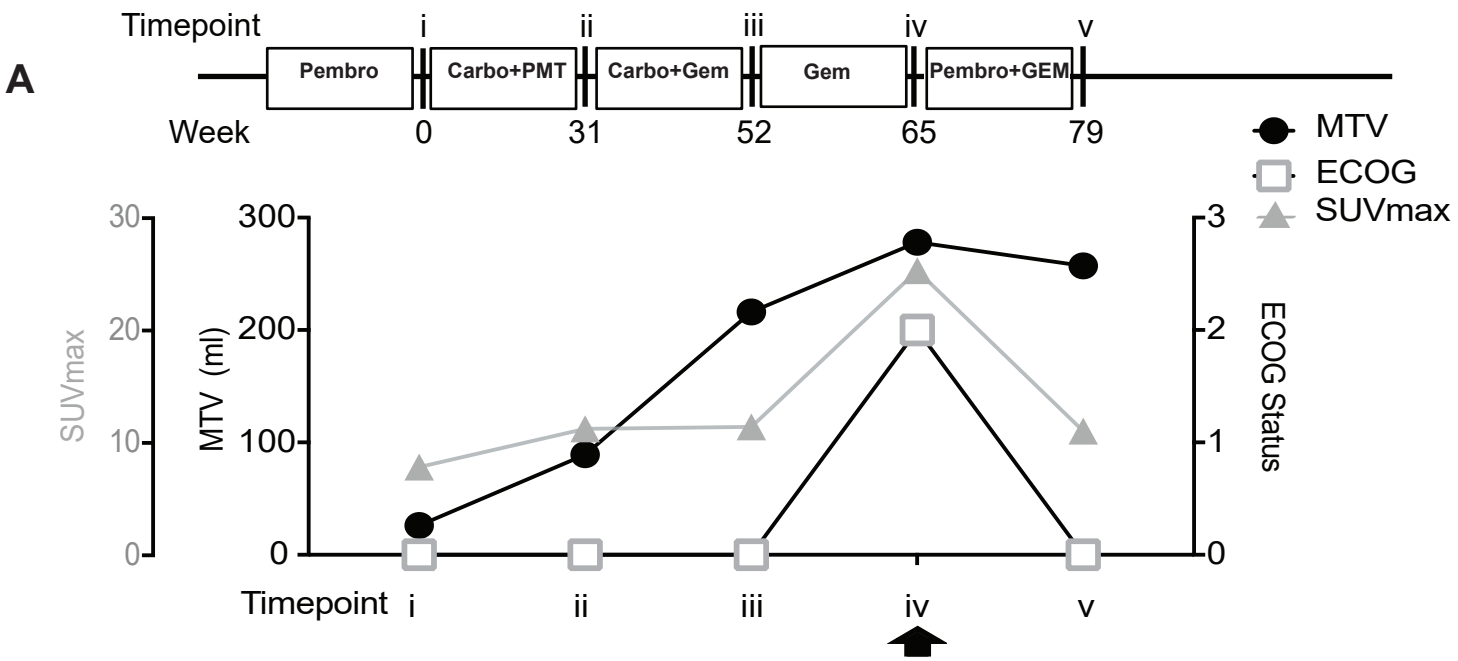


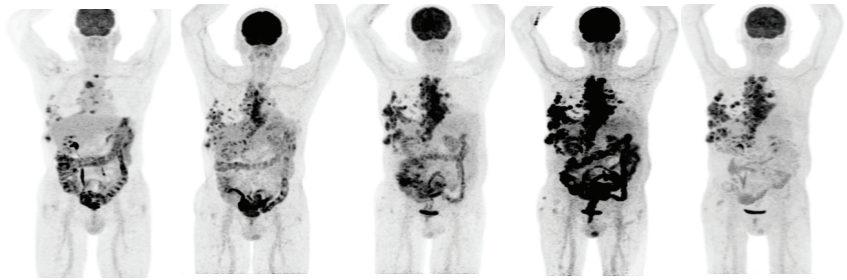
Tallón de Lara et al. Fig. 3

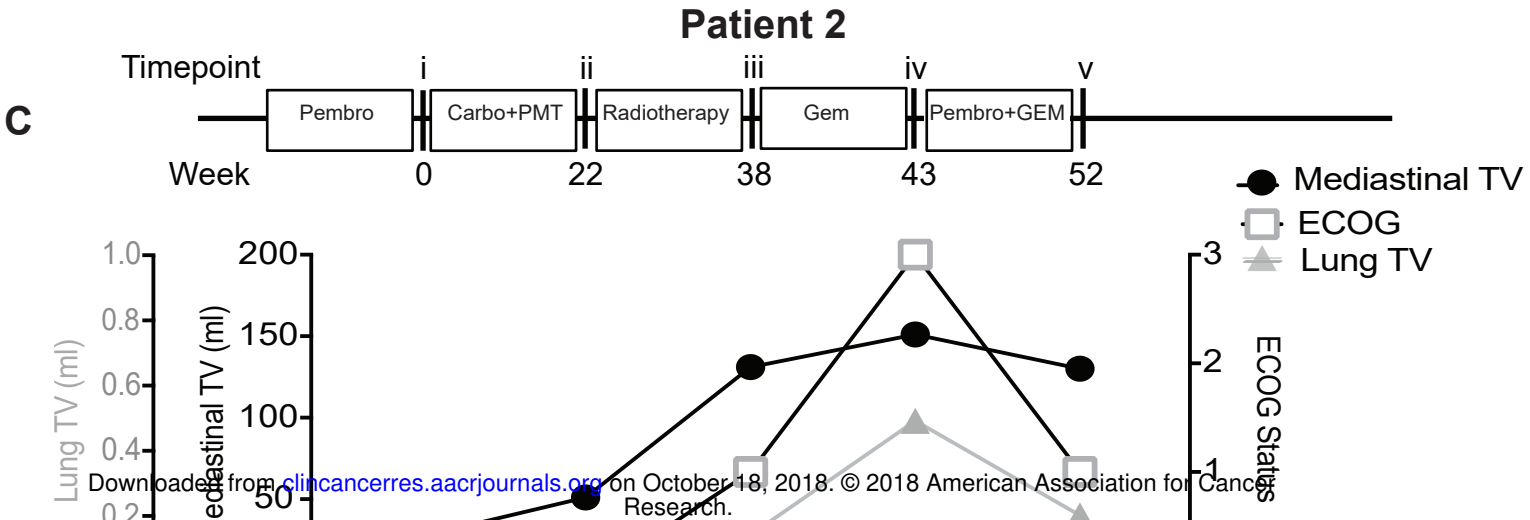
Author Manuscript Published OnlineFirst on August 28, 2018; DOI: 10.1158/1078-0432.CCR-18-1231
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

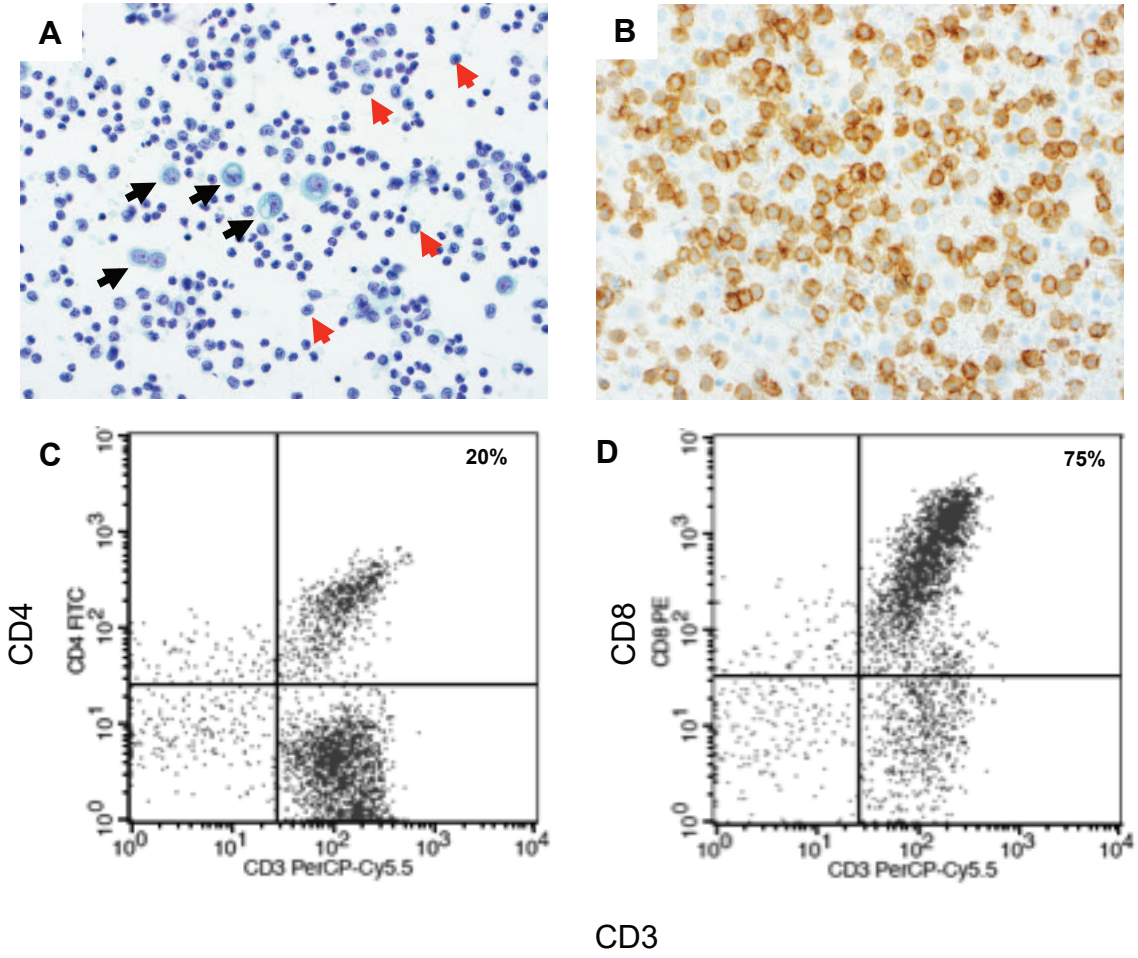


Tallón de Lara et al. Fig. 4









Tallón de Lara et al. Fig. 6

AACR American Association for Cancer Research

Clinical Cancer Research

Gemcitabine synergizes with immune checkpoint inhibitors and overcomes resistance in a preclinical model and mesothelioma patients

Paulino Tallon de Lara, Virginia Cecconi, Stefanie Hiltbrunner, et al.

Clin Cancer Res Published OnlineFirst August 28, 2018.